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13. SUPPLEMENTARY NOTES

14. ABSTRACT

During 2008-09 funding period we made considerable progress on our grant proposal and published one paper in Molecular Cancer Therapeutics. Our research findings were considered for highlight and appeared on cover page (cover illustration, MCT September 2008, volume 7). Along with publication three more papers were published from the lab. In next funding cycle we are expected to make progress on remaining specific aims and to have couple more publications. In brief, in 2008-09 funding period we have investigated the effect of bryostatin on PKD1 expression, beta-catenin transcription, cell proliferation, and cellular aggregation. In this study we examined the effect of Bryostatin 1 treatment on PKD1 activation, β-catenin translocation and transcription activity and malignant phenotype of prostate cancer cells. Initial activation of PKD1 with Bryostatin 1 leads to colocalization of the cytoplasmic pool of β-catenin with PKD1, trans-Golgi network markers and proteins involved in vesicular trafficking. Activation of PKD1 by Bryostatin 1 decreases nuclear β-catenin expression and β-catenin/TCF transcription activity. Activation of PKD1 alters cellular aggregation and proliferation in prostate cancer cells associated with subcellular redistribution of E-cadherin and β-catenin. For the first time, we have identified Bryostatin 1 modulates β-catenin signaling through PKD1, which identifies a novel mchansim to improve efficacy of Bryostatin 1 in clinical setting.

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New investigator Award Proposal

Modulation of β-catenin activity with PKD1 in Prostate Cancer (PC 073643)

(PI: MEENA JAGGI)

INTRODUCTION

Understanding the basic biology of prostate cancer will provide us with additional critical information necessary to improve existing treatments or to find newer treatments for patients suffering from prostate cancer. While several genetic alterations play specific functions in a cell, some of these genes can have multiple functions. One such gene encodes a protein called β-catenin, which plays a dual role in cancer cells by: a) playing a role in cellular adhesion via another protein called E-cadherin, and b) playing a role in causing cellular division through a set of proteins which cause cells to divide abnormally. In addition to these proteins which β-catenin is already known to associate with, we have discovered a new interaction with a protein called protein kinase D1 (PKD1) in prostate cancer cells. Unraveling this complex interaction of β-catenin with PKD1 in prostate cancer cells may hold the key to understanding the role of a single important protein in causing unregulated cellular division and loss of cellular adhesion – the two fundamental hallmarks of a cancer cell. We have previously made two important discoveries in this field: a) PKD1 levels are lower in advanced prostate cancer which are associated with more aggressive types of cancer, and b) PKD1 interacts with another important protein in cancer cells, β-catenin. These preliminary discoveries in prostate cancer have led us to put forth the current proposal. Our major objective in this proposal is to understand the consequences of binding of PKD1 to β-catenin in tumor development and to study the exact alteration of these proteins in human prostate cancer tissues.

Understanding the details of how cancer causing proteins communicate with each other in a cell will help us intervene in the disease process more effectively. To this end, we propose to study the effect of β-catenin and PKD1 interaction on the cancer cell. We plan to achieve these goals by increasing PKD1 activity in the cell by use of a drug called Bryostatin1, which has already been used in clinical trials in various types of cancers. During 2008-09 funding period we made considerable progress on our grant proposal and published one paper in Molecular Cancer Therapeutics. Our research findings were considered for highlight and appeared on cover page (cover illustration, MCT September 2008, volume 7). Along with publication three more papers were published from the lab. In brief, we have investigated the effect of bryostatin on PKD1 expression, β-catenin transcription, cell proliferation, and cellular aggregation. In this study we examined the effect of Bryostatin 1 treatment on PKD1 activation, β-catenin translocation and transcription activity and malignant phenotype of prostate cancer cells. Activation of PKD1 with Bryostatin 1 leads to colocalization of the cytoplasmic pool of β-catenin with PKD1, trans-Golgi network markers and proteins involved in vesicular trafficking. Activation of PKD1 by Bryostatin 1 decreases nuclear β-catenin expression and β-catenin/TCF transcription activity. Activation of PKD1 alters cellular aggregation and proliferation in prostate cancer cells associated with subcellular redistribution of E-cadherin and β-catenin. For the first time, we have identified Bryostatin 1 modulates β-catenin signaling through PKD1, which identifies a novel mchansim to improve efficacy of Bryostatin 1 in clinical setting.

BODY

Aim 1: Molecular Nature of PKD1 and β-catenin Interaction

To determine domain of PKD1 that are required for interaction with β-catenin:

We have generated stable clones of C4-2 cells with green fluorescent protein (GFP) fused PKD1 constructs in order to study the physiological significance of PKD1 in prostate cancer. Fluorescence activated cell sorting (FACS) was used to select for homogeneous populations of stably transfected cells expressing PKD-GFP utilizing the Flow Cytometry. These homogeneous populations will be analyzed to determine PKD1 mutant construct interactions with β-catenin and subcellular localization by confocal immunofluorescence microscopy.

Generation of PKD1 mutant constructs and generation of C4-2 stable cell lines expressing PKD–GFP construct:

The DNA constructs of PKD1 mutants tagged with GFP and cloned in pEGFP vector were obtained from our collaborator Dr. Angelika Hausser, University of Stuttgart, Germany. Stable C4-2 prostate cancer cell lines containing the mutant PKD constructs were generated by chemical transfection using Lipofectamine2000. The cells were observed for GFP under a fluorescent microscope (Fig. 1). After two days of incubation, the cells were expanded into a 100mm plate and selection pressure was applied to these C4-2 cells, for selection of stably transfected cells, by replacing the media with 300ug/ ml G418 (geneticin) containing media. The cells were allowed to grow at 37°C/5%CO_2 with regular replacement of fresh selection media and intermittently checked for selection and formation of fluorescent C4-2-GFP colonies. In next cycle the interaction of PKD1-GFP mutant constructs with β-catenin will be analyzed by immunoprecipitation assays, using β-catenin and GFP specific antibodies as described previously. Additionally, the effect of the different constructs on β-catenin transcription will be analyzed.

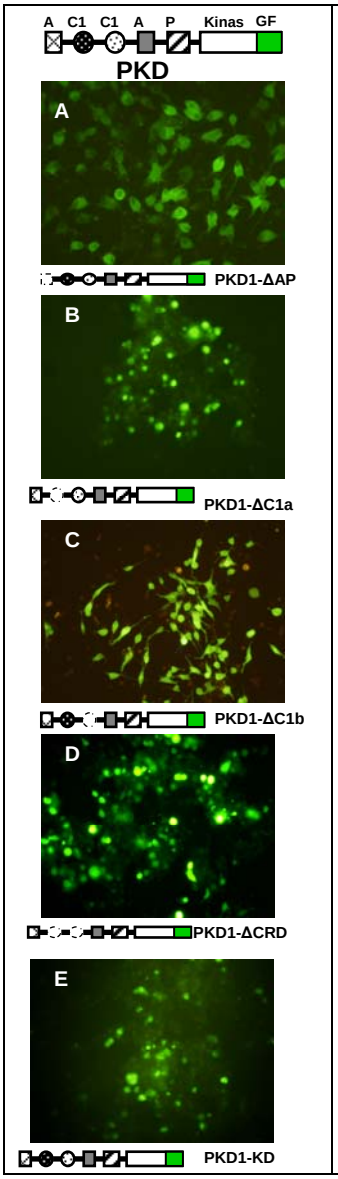

Figure 1. Generation of stable C4-2-PKD1 mutant cell lines:

Stable C4-2-PKD1 mutant cell lines were generated using transfecting agent Lipofectanmine2000. The figure above shows fluorescent microscopic images of transfected cells and a pictorial representation of the construct used. (A). C4-2 cells transfected with AP domain deleted PKD1 (C4-2-PKD1-ΔAP). (B). C4-2 cell stransfected with C1a domain deleted PKD1 (C4-2-PKD1-ΔC1a). (C). C4-2 cells transfected with C1b domain deleted PKD1 (C4-2-PKD1-ΔC1b). (D). C4-2 cells transfected with C1a and C1b domains deleted from PKD1 (C4-2-PKD1-ΔCRD). (E). C4-2 cells transfected with kinase dead (K612W) PKD1 (C4-2-PKD1KD).

Aim 2: To Demonstrate that Activation of PKD1 by Bryostatin1 Influences the Cellular Phenotype in Prostate Cancer.

Time and dose dependent activation of PKD1 by Bryostatin 1

Treatment of the prostate cancer cells stably transfected with PKD1-GFP with increasing concentrations (10-30 nM) of Bryostatin 1 for 3 h demonstrated increased transphosphorylation of ser738 and ser742 and autophosphorylation of ser910 residues of PKD1 (Fig. 2.1 A and B). The activation of PKD1 is phosphorylation-dependent, and serine738 and 742 residues in human PKD1 (corresponding to serine744 and 748 in mouse) have been identified as crucial phosphorylation sites. These serine residues are located in the activation loop of the PKD1 catalytic domain. The C-terminal serine916 residue has been identified as an autophosphorylation site in PKD1 (1). Phosphorylation of these serine residues affects PKD1 activity and plays a role in modulation of PKD1 function *in vivo*. In order to exclude cell line specific effects we also confirmed that Bryostatin 1 activated and is associated with membrane translocation of PKD1 in androgen dependent LNCaP cells (data not shown).

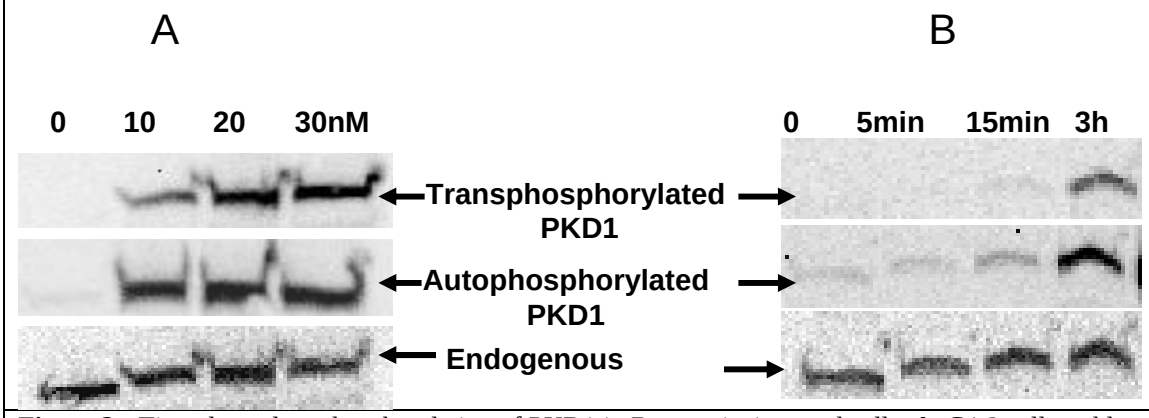

Figure 2: Time dependent phosphorylation of PKD1 in Bryostatin 1 treated cells, **A**, C4-2 cells stably transfected with GFP fused PKD1 (C4-2-PKD1-GFP) were treated with 0, 10, 20 and 30 nM Bryostatin 1. Cell extracts were immunoblotted with auto- and transphosphorylation site specific antibodies. Note optimum phosphorylation at 10 nM. **B**, C4-2 -PKD1-GFP cells were treated with 10 nM Bryostatin 1 for 0-3 h. Cell extracts were immunoblotted with auto- and transphosphorylation site specific antibodies. Note maximum phosphorylation at 3 h.

Effect of activation of PKD1 by Bryostatin 1 on E-cadherin and β-catenin subcellular localization

Subcellular localization of PKD1, E-cadherin and β-catenin in Bryostatin 1 activated C4-2-PKD1-GFP cells was analyzed by confocal microscopy. To examine PKD1 specific changes in subcellular localization of E-cadherin and β-catenin, we compared E-cadherin and β-catenin localization in Bryostatin 1 activated C4-2-GFP cells and C4-2-PKD1-GFP cells. In vector transfected C4-2 cells we did not detect any change in E-cadherin or β-catenin localization after Bryostatin 1 activation (Fig. 3.1). Our immunofluorescence study clearly revealed perinuclear and membrane localization of PKD1-GFP upon activation by Bryostatin 1 (Fig. 3.2). The most striking observation was the colocalization of E-cadherin and β-catenin with PKD1-GFP in Bryostatin 1 activated C4-2-PKD1-GFP cells at perinuclear areas in addition to cell membranes (Fig. 3.2, arrows). After 24 h of Bryostatin 1 treatment, strong membrane staining of E-cadherin/β-catenin and some perinuclear staining was also noticed (Fig. 3.3). While the C4-2-GFP cells do not over-express PKD1-GFP (Fig. 3.1), they do not show perinuclear localization

of E-cadherin/β-catenin. This observation confirms that E-cadherin/β-catenin subcellular distribution is specifically mediated by PKD1 activation and not by other kinases activated by Bryostatin 1.

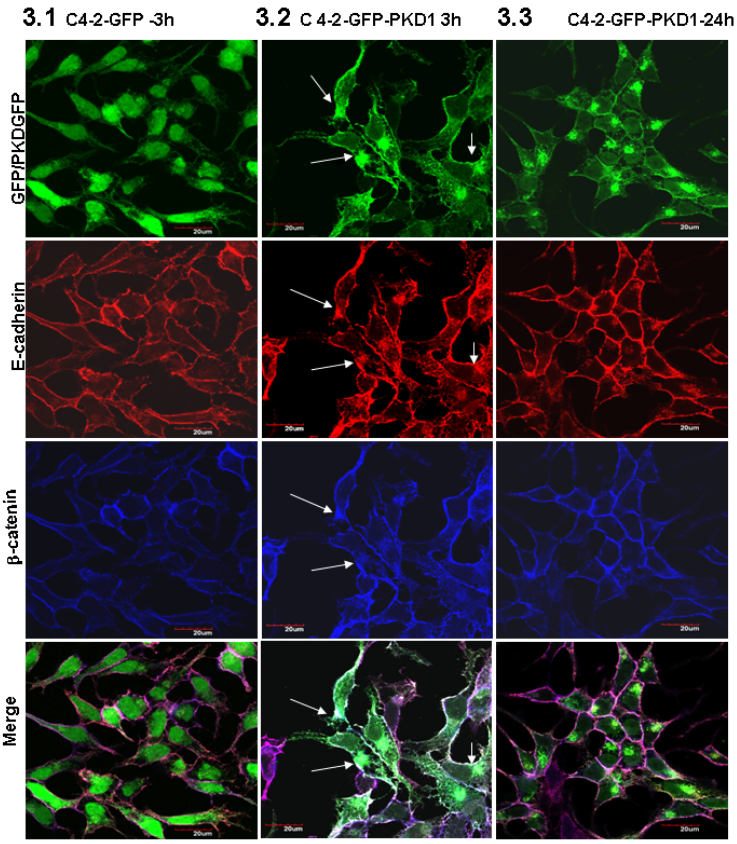

Figure 3: Activation of PKD1 by Bryostatin 1 on E-cadherin and β-catenin subcellular localization. Bryostatin 1 activated C4-2-GFP and C4-2-PKD1-GFP cells were stained for E-cadherin and β-catenin and analyzed by LSM. C4-2-GFP (**3.1**) and C4-2-PKD1-GFP (**3.2** and **3.3**) cells show differences in subcellular localization of E-cadherin (red) and β-catenin (blue). E-cadherin and β-catenin colocalizes with PKD1-GFP at perinuclear areas in addition to cell membranes (**3.2** arrows) in Bryostatin 1 activated C4-2-PKD1-GFP cells (**3.2** and **3.3**) but not in C4-2-GFP cells.

Bryostatin treatment decreases β-catenin transcriptional activity

We investigated the effect of PKD1 activation on β-catenin mediated transcription activity and proliferation in prostate cancer cells. To investigate the effect of PKD1 on β-catenin mediated transcription activation of TCF, we transfected plasmids containing a wild type TCF-binding site (TOPFlash) or a mutated site as a negative control (FOPFlash) with pRL-TK (*Renilla* luciferase) in C4-2-PKD1-GFP cells activated with Bryostatin 1 or DMSO. The firefly and *Renilla* luciferase activities were measured with the Dual-Luciferase Reporter (DLR) Assay System. After normalizing the firefly luciferase activity to that of *Renilla* luciferase, the FOPFlash reporter plasmid luciferase values were subtracted from the normalized values obtained with the TOPFlash reporter plasmid. Bryostatin 1 activation in C4-2-PKD1-GFP cells led to a significant reduction (p value=0.019) in β-catenin reporter activity (Fig. 4.1).

Effect of activation of PKD1 by Bryostatin 1 proliferation

The cell proliferation ability of Bryostatin 1 activated C4-2-PKD1-GFP cells was assayed by CellTiter-Glo. Bryostatin 1 activated C4-2-PKD1-GFP cells showed a 40% decrease in cell proliferation as compared to DMSO treated cells (Fig. 4.2). A mixed ANOVA model will be used to compare the cell lines and doses. P-value < 0.05 was considered significant.

Effect of activation of PKD1 by Bryostatin1 on cellular aggregation

PKD1 is also known to be involved with altered cellular aggregation, which is required for a cancer cell to successfully complete the metastatic cascade (2). Because we have demonstrated that PKD1 activation with Bryostatin 1 is involved in trafficking of β-catenin, we sought to determine the effect of Bryostatin 1 activation on cellular aggregation in C4-2 cells over-

expressing PKD1. Aggregation assays were performed on C4-2 cells expressing PKD1-GFP as described previously. Our experiments demonstrated increased cellular aggregation in Bryostatin 1 treated C4-2-PKD1-GFP cells compared to vehicle only treated cells (Fig. 4.3).

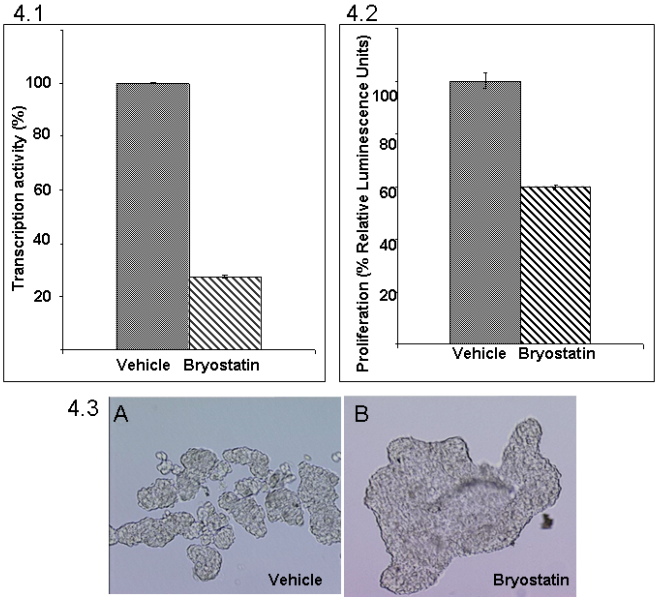

Figure 4: Bryostatin treatment decreases in β-catenin transcription activity, cell proliferation and increases cell-cell adhesion. **4.1)** Effect of Bryostatin treatment on β-catenin transcription activity**.** C4-2-PKD1-GFP cells were transfected with TOP or FOPFlash firefly luciferase reporter constructs and treated with 10 nM Bryostatin 1 or vehicle only. Data are expressed as fold induction normalized to the cotransfected Renilla luciferase-encoding pRL-TK plasmid. Treatment of C4-2-PKD1-GFP cells significantly decreased β-catenin mediated transcription activity as compared to vehicle only treated cells ($p = 0.019$). Error bars indicate standard error. **4.2)** Proliferation assay of Bryostatin 1 treated C4-2-PKD1-GFP cells. Proliferation assay after 48 h of 10 nM Bryostatin 1 treatment (see Materials and Methods) expressed as percent of control cells treated with vehicle only ($p=0.001$). Error bars indicate standard error. **4.3)** Bryostatin treatment increases cellular aggregation. Bryostatin 1 treated C4-2 -PKD1-GFP cells showed increased cellular aggregation (**B**) compared to vehicle only treated cells (**A**).

Effect of PKD1 inhibition on β-catenin subcellular localization

To further demonstrate the specific function of PKD1 in mediating the subcellular redistribution of β-catenin, PKD1 expression was inhibited 90% by using small interfering RNA (siRNA) in C4-2-PKD1-GFP cells activated with Bryostatin 1. After inhibition of PKD1 expression, cells transfected with non-targeted siRNA were activated with Bryostatin 1, stained for β-catenin and trans Golgi network (TGN) specific (p230) antibody and analyzed by confocal microscopy. Non-targeted siRNA transfected and Bryostatin 1 activated cells showed perinuclear localization of PKD1 and β-catenin. Merging of PKD1, β-catenin and p230 images from these cells shows colocalization of these three proteins at the perinuclear region and colocalization of PKD1 and β-catenin at the cell junction. Immunofluorescence images of PKD1 siRNA transfected C4-2-PKD1-GFP cells shows inhibition of PKD1-GFP (Fig. 5.1), reduced staining of β-catenin at the membrane and lack of β-catenin localization at TGN (Fig. 5 F). Merging of PKD1, β-catenin and p230 images taken at the same confocal level in PKD1 siRNA transfected C4-2-PKD1-GFP cells (Fig. 5 H) do not show colocalization of the proteins at the perinuclear region. These results suggest that β-catenin subcellular localization is modulated predominantly by activated PKD1 and not by other kinases (PKC isoforms) activated by Bryostatin 1. Interestingly, down regulation of PKD1 by RNAi decreased β-catenin expression at the plasma membrane (Fig 5.2), which further suggests that PKD1 plays a major role in membrane transport of β-catenin. We have previously published that down regulation of PKD1 in fact increases total cellular β-catenin. This provides further corroborative evidence for role of PKD1 in membrane trafficking

of β-catenin because membrane β-catenin is decreased in spite of increased total levels of cellular β-catenin when PKD1 expression is reduced (3). However, the exact mechanism of regulation of β-catenin expression by PKD1 remains to be investigated.

Effect of PKD1 expression on apoptosis:

It has been shown that nuclear β-catenin forms a complex with TCF/LEF transcription factors and that this complex transactivates downstream targets such as *c-myc* and *cyclin D1*. These proteins have been implicated in cell cycle regulation. PKD1 overexpression in C4-2 cells decreases β-catenin/TCF transcription activity. Over expression of PKD1 causes increased cellular aggregation and decreased motility in prostate cancer cells. In order to determine the effect of PKD1 on cell cycle distribution Cell cycle distribution was assessed using BD FACSVantage SE pulse processing plus program for analysis of DNA content. C4-2-GFP vector and C4-2-GFP-PKD1 cells were stained with propidium iodide (PI). Each value represents percentage of cells in the noted cell cycle phase. Experiments were repeated three times and representative histograms are shown. The results show that PKD1overexpression resulted increase of cells in G1 phase and concomitant decrease in cell in G 2 phase, indicate cell cycle arrest in G1 phase.

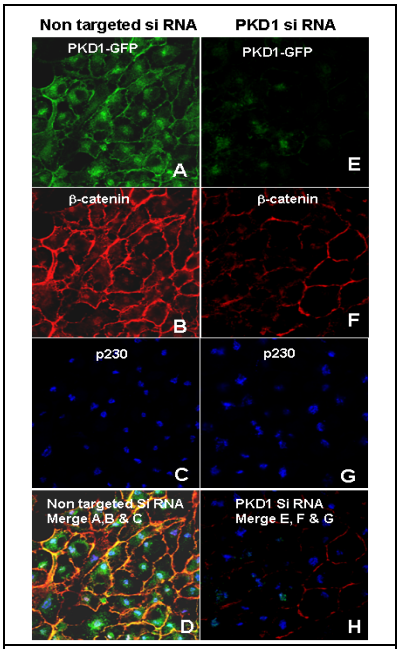

Figure 5: Inhibition of PKD1 on β-catenin subcellular localization. Bryostatin 1 treated C4-2-PKD1-GFP cells were transfected with a PKD1 silencing siRNA and stained for β-catenin and p230 antibodies. β-catenin shows strong membrane localization and perinuclear localization in control siRNA transfected cells, whereas PKD1 silenced cells showed punctuate membrane staining and does not show perinuclear staining.

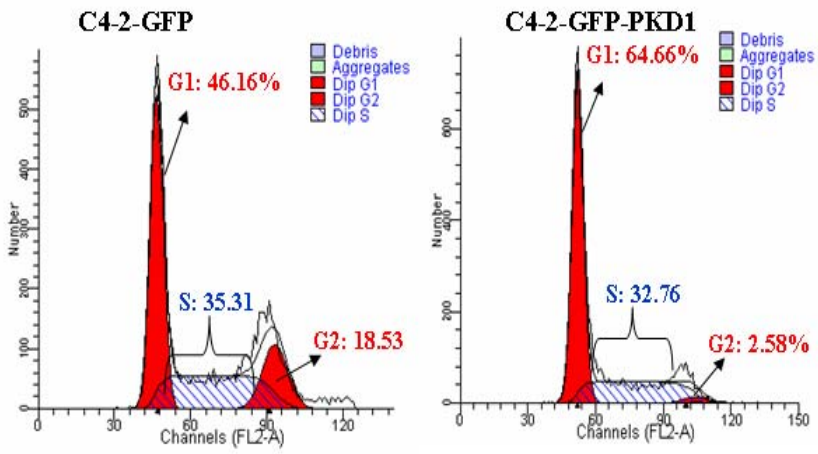

Figure 6: Cell cycle analysis: C4-2-GFP vector and C4-2-GFP-PKD1 cells were stained with propidium iodide (PI) and cell cycle distribution was assessed by fluorescence-activated cell sorting (FACS) analysis of DNA content. Each value represents percentage of cells in the noted cell cycle phases .

Plan for next year:

Effect of PKD1 mutant constructs* β-catenin *transcription:

We will investigate the effect of PKD1 mutants on β-catenin mediated transcriptional activity and proliferation in prostate cancer cells. To investigate the effect of PKD1 mutants on β-catenin mediated transcriptional activation of TCF, we transfected plasmids containing a wild type TCF-binding site (TOPFlash) or a mutated site as a negative control (FOPFlash) with pRL-TK (*Renilla* luciferase) in C4-2-PKD1-GFP cells activated with Bryostatin 1 or DMSO. The firefly and *Renilla* luciferase activities were measured with the Dual-Luciferase Reporter (DLR) Assay System. After normalizing firefly luciferase activity to that of *Renilla* luciferase, the FOPFlash reporter plasmid luciferase values were subtracted from the normalized values obtained with the TOPFlash reporter plasmid. This study will determine site specific functions of PKD1 in β-catenin mediated cellular signaling.

Effect of activation of PKD1 by Bryostatin1 on DNA damage induction and apoptotic cell death

Both parental C4-2 cells and C4-2 cells transfected with PKD1 will be seeded on coverslips, cultured for 24 hours, and treated with Bryostatin1 at multiple time points (5, 10, 15 minutes, etc.) to study the rate of apoptotic cell death. Apoptosis will be assayed by TdT-mediated nick end labeling (TUNEL) by using an in situ cell death detection kit (Promega, Madison, WI). The rate of apoptosis of the treated cells will be compared to the controls.

Modulation of the tumorigenicity of prostate cancer cells by PKD1

The ability of PKD1 to alter the growth potential of prostate cancer cells will be assayed by the classic transformation parameter of anchorage-dependent growth in soft agar. The assay will be conducted as previously described (4) with some modifications. Briefly, $2.5x10^2$ cells will be suspended in 1ml RPMI containing a final concentration of 0.4% agar and 10% FBS. The cells will be plated on top of a solidified layer of 0.6% agar in six well plates. The numbers of colonies will be scored at day 15 and photographed using a phase microscope. In addition to in vitro studies we will perform in tumorigenicity assay using mouse xenograft model. Male athymic Swiss Webster nude mice, aged 6 to 8 weeks will be obtained from Jackson laboratories. Prostate cancer cells with vector, PKD1 over-expresing and PKD1 silenced will be used for tumor development. At the end of the experiments all tumors will be dissected, measured, weighed and specimen saved at -80^oC for correlative studies.

Effect of PKD1 expression influences motility and invasion of prostate cancer cells

The effect of PKD1 expression on motility will be studied by *in vitro* motility assays as described in our prior publication (2). We anticipate increased cell motility in the PKD1 knockdown as compared to controls.

KEY RESEARCH ACCOMPLISHMENTS

During 2008-09 funding period we made considerable progress on our grant proposal and published one paper in Molecular Cancer Therapeutics. Our research findings were considered for highlight and appeared on cover page (cover illustration, MCT September 2008, volume 7). Along with publication three more papers were published from the lab. In next funding cycle we are expected to make progress on remaining specific aims and to have couple more publications.

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***Publications from the grant* (:**

Jaggi M*., Chauhan SC., Du C. and Balaji KC**.** Bryostatin modulates β-catenin subcellular localization and transcription activity through protein kinase D1 activation. **Molecular Cancer Therapeutics** 2008;7(9):2703-12 **(Cover illustration)**

Total publications (2008-2009):

1. Chauhan SC., Vannatta K., Ebeling MC., Vinayek N., Watanabe A., Pandey KK., Maher D., Bell MC., Koch MD., Aburatani H., Lio Y. and **Jaggi M.** Expression and *in vitro* functions of transmembrane mucin MUC13 in ovarian cancer. *Cancer Research* 2009;69(3) 765-774
2. **Jaggi M#**., Du C#., Zhang C. and Balaji KC. Protein kinase D1 (PKD1) mediated phosphorylation and subcellular localization of β-catenin. *Cancer Research* 2009;69(3) (-# equal contribution)
3. **Jaggi M***., Chauhan SC., Du C. and Balaji KC**.** Bryostatin modulates β-catenin subcellular localization and transcription activity through protein kinase D1 activation. **Molecular Cancer Therapeutics** 2008;7(9):2703-12 **(Cover illustration)**
4. Paul M., **Jaggi M**., Viqar S., Chauhan SC., Hassan S., Biswas H., Balaji K.C. Protein kinase D1 (PKD1) influences androgen receptor (AR) function in prostate cancer cells. *Biochemical and Biophysical Research Communications* 2008 Sep 373:618-23

REPORTABLE OUTCOMES/CONCLUSIONS

- Published one paper in Molecular Cancer Therapeutics. Our research findings were considered for highlight and appeared on cover page (cover illustration, MCT September 2008, volume 7).
- Bryostatin 1 treatment modulates PKD1 expression, cell proliferation, and cellular aggregation.
- Bryostatin 1 treatment alters β-catenin translocation and transcription activity.
- Initial activation of PKD1 with Bryostatin 1 leads to colocalization of the cytoplasmic pool of β-catenin with PKD1, trans-Golgi network markers and proteins involved in vesicular trafficking.
- Activation of PKD1 by Bryostatin 1 decreases nuclear β-catenin expression and thereby suppresses β-catenin/TCF transcription activity.
- For the first time, we have identified Bryostatin 1 modulates β-catenin signaling through PKD1.

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APPENDICES

N/A